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Is the chromanol head group of vitamin E nature's final truth on chain-breaking antioxidants?

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ABSTRACT

Tocopherol is believed to be the most potent naturally occurring chain-breaking antioxidant. Hence, its refined phenolic head group chromanol may represent an optimum evolutionary solution to the problem of free-radical chain reactions in the lipid bilayer. To test the universal validity of this assumption beyond phenolic head groups, we have synthesized aromatic amine analogues of vitamin E and trolox with otherwise closely matching physicochemical properties: NH-toc and NH-trox. We have found that NH-toc and NH-trox were significantly more potent free radical scavengers, lipid peroxidation inhibitors and cytoprotective agents than their phenolic templates, tocopherol and trolox. In a chemical sense, thus, the chromanol head group does not constitute a global optimum for the design of chain-breaking antioxidants.

1. Introduction

Tocopherols and tocotrienols (vitamin E) are among the most potent chain-breaking antioxidants known [1], and they play a central role for the functionality of a variety of cells and tissues, such as cerebellar, peripheral, and retinal neurons [2,3]. Tocopherol is also the most effective natural antioxidant to protect cultivated neuronal cells from oxidative cell death [4,5]. Hence, tocopherol can translate its high in vitro activity into the in vivo situation, whereas many other radical scavengers partially or fully lose their apparent in vitro activity when tested in intact cells, for example spin traps [4–7].

It has been hypothesized that tocopherol's chromanol head group may reflect an optimum evolutionary answer to the threat of toxic free-radical chain reactions in the lipid bilayer. This conclusion has primarily been drawn from favourable comparisons with other phenols in vitro, in vivo and in silico [1,4,5,8–12]. Even sophisticated approaches to the optimization of the chromanol head group [12,13] and elaborate de novo strategies [14,15] have yielded only modestly more potent structures. Moreover, the reported higher reactivities of certain dihydrobenzofuranols [12] or pyridinols [14] towards peroxyl radicals were achieved by compounds with a significantly smaller molecular weight and a correspondingly higher diffusion coefficient than tocopherol, suggesting that this factor may have been a primary cause of the observed improvement. This possibility is relevant as smaller compounds will generally not achieve the same favourable distribution and retention in cells and tissues as tocopherol does [16], potentially nullifying the compiled in vitro advantage. Apparently, in no case has a compound of the same, relatively high molecular weight as tocopherol been reported to be more potent than the latter in terms of its EC_{50} value in a biologically relevant peroxidation assay.

In consequence of tocopherol's unsurpassed efficacy in many experimental settings, its chromanol head group has frequently been favoured as template for the development of intentionally smaller (and thus more diffusible), more hydrophilic (and thus not membrane-restricted), or otherwise tailored artificial antioxidant substances [12,13,17,18]. Specifically, the amphiphilic, carboxylated chromanol compound trolox [17] has become a widely employed standard for the assessment of free radical scavenging activities in terms of the "trolox equivalent antioxidant capacity" (TEAC) assay [19].

In an effort to expand the chemical space of hydrogen radical-donating antioxidants, we have investigated whether the substitution of oxygen in phenolic compounds by nitrogen might be associated with an improved stability of transiently formed antioxidant radicals, especially as amines offer the potential to add two aromatic rings to the primary site of radicalization (Fig. 1A). Aromatic amines, especially secondary amines with bridged aromatic

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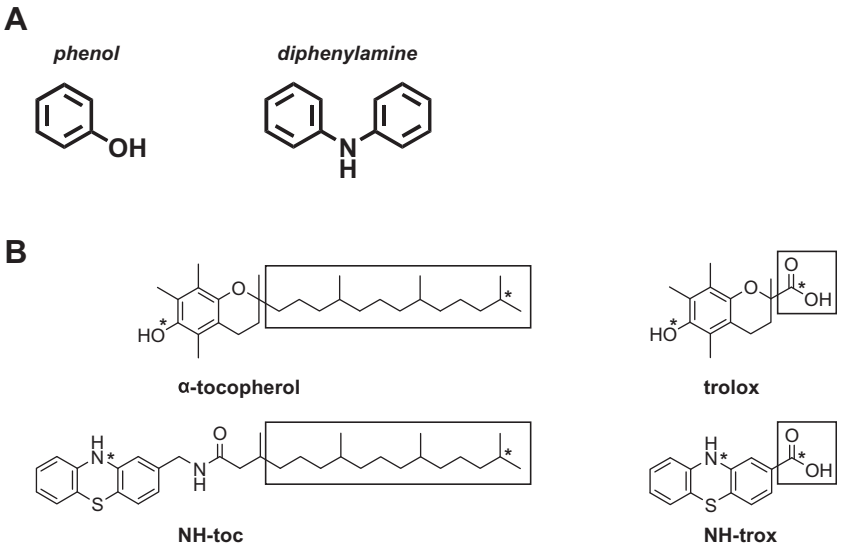

Fig. 1. Chemical structures. (A) The chemical structures of phenol and diphenylamine, illustrating that amines can form bonds to two independent aromatic systems while retaining a homolytically dissociable NH bond. (B) The chemical structures of α-tocopherol, trolox, NH-toc, and NH-trox. Boxes indicate the pairwise identical solubility-determining side-chains; the remainder of each molecule was defined as its head group. Asterisks label the atoms that were chosen for molecular distance calculations.

substituents, are known to be potent antioxidants [20]. They possess a very versatile redox biochemistry [21], and similarly as tocopherol, they can translate their antioxidant activity in vitro into the in vivo situation [22]. For example, aromatic amines have recently been shown to afford complete and receptor-independent antioxidant protection against dopaminergic neurodegeneration in *Caenorhabditis elegans* nematodes treated with the prooxidative complex I inhibitors rotenone and MPP^+ [23]. In the following, we thus report on the synthesis of two novel, amine-based head group analogues of trolox and tocopherol, and we evaluate their antioxidant potential.

2. Materials and methods

2.1. Chemicals

All chemicals and biochemicals were from Sigma–Aldrich if not otherwise indicated. Cell culture reagents were from Invitrogen. 2-Cyano-phenothiazine was obtained from Bernhagen-Chemie; phytanic acid was from Cayman Chemicals. Synthetic (±)-α-tocopherol (purity 97%) and natural (+)-γ-tocopherol (purity 99%) were from Sigma–Aldrich.

2.2. Synthesis and computational evaluation of NH-toc and NH-trox

The synthesis of NH-toc (Fig. 1) was achieved by reduction of 2-cyano-phenothiazine to 2-aminomethyl-phenothiazine with $LiAlH_4$ in dichloromethane at RT, following a standard protocol [24]. The resulting primary amine was then coupled with phytanic acid using ethylchloroformate/*N*,*N*-diisopropylethylamine in −20 °C DMF essentially as published [25]. The compound NH-trox (Fig. 1) was synthesized from 2-cyano-phenothiazine by hydrolysis in refluxing concentrated HCl/acetic acid according to customary protocols [26]. All compounds were purified by precipitation and re crystallization, or by preparative column chromatography. Their identity and purity were verified by ^1H NMR spectroscopy, mass spectrometry, and analytical thin-layer chromatography.

Molecular calculations yielding log*P* values and molecular distances were performed with the ChemDraw/Chem3D software package (CambridgeSoft). Semi-empirical quantum chemical calculations to determine heats of formation, orbital energies and dipolar moments were accomplished with the implemented Chem3D MOPAC routine, using the AM1 Hamiltonian as described [20].

2.3. Assessment of the antioxidant activity of NH-toc and NH-trox

The in vitro ability of the investigated compounds to react with free radicals was determined by measuring their quenching of preformed 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals in aqueous and ethanolic solution. The results were expressed as trolox equivalent antioxidant capacities (TEACs) as described [27].

The biochemical activity of NH-toc and NH-trox to prevent lipid peroxidation was investigated in a native preparation of highly unsaturated rat brain membranes [10]. Lipid peroxidation was induced by treatment with iron/ascorbate or iron/H_2O_2 and was quantified by measuring (i) end products of polyunsaturated fatty acid degradation (thiobarbituric acid-reactive substances, TBARS) and (ii) the specific formation of 8-iso prostaglandin $F_{2\alpha}$ (8-iso-prostane) from arachidonic acid. TBARS were measured exactly as described [22], 8-isoprostanes were analyzed by enzyme immunoassay (Cayman Chemicals).

The protection of cultivated murine clonal hippocampal neurons (HT22 cells) from oxidative glutamate toxicity [28] was employed to assess the in vivo antioxidant activity of the compounds under investigation. Cell cultivation and propagation as well as the analysis of cell survival and cell viability were done as detailed [20]. Two independent methods to assess cellular survival and viability were employed, (i) the metabolic reduction of the 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazolium (MTT) cation towards a coloured formazan, which is exclusively afforded by viable cells, and (ii) the exclusion of the fluorescent DNA-intercalating dye propidium iodide (PI) from cells with intact plasma membrane. Quantification was achieved by photometric analysis (MTT test) and by visual inspection and counting of intact and damaged cells under the microscope (PI test).

2.4. Cellular incorporation of NH-toc and NH-trox

An HPLC routine was employed to monitor the relative cellular incorporation of the novel compounds. HT22 cells treated with the

compounds under investigation were cultivated as in the cytoprotection experiments. At the indicated time, the medium was removed, and the cells were harvested mechanically in a fresh aliquot of cultivation medium. For the analysis of tocopherol and NH-toc, the samples were vigorously extracted with 4 vol of methanol at 4 °C, followed by centrifugation at 7500×*g* for 20 min. The upper, clear phase was directly injected onto a C18 reversed-phase column and eluted with methanol:water (96:4, v/v). Tocopherol was detected by its fluorescence at 296/340 nm, NH-toc was traced via its absorption at 254 nm. For the analysis of trolox and NH-trox, the aqueous phases were acidified with 0.05 vol of 6 M HCl before extraction with 3 vol of ether. The ether phase was vacuum dried, and the residue was dissolved in a small volume of methanol. Immediately before injection onto a C18 reversed-phase column, the methanolic solution was mixed 1:1 (v/v) with mobile phase, consisting of methanol:water:acetonitrile:acetic acid (47:47:5:1, v/v/v/v) adjusted to 20 mM phosphoric acid. Trolox was quantified at 292 nm excitation/327 nm emission, NH-trox was monitored at 254 nm.

3. Results

3.1. Design and computational characterization of NH-toc and NH-trox

In order to assess in how far the chromanol head group of tocopherol was functionally optimized, we have attempted to design and synthesize head group analogues of tocopherol (and trolox) with as little difference as possible to the corresponding parental structures regarding the relevant size and solubility parameters, yet with as much difference as possible regarding the electronic nature and radicalization behaviour of the newly introduced hydrogen-donating head group. The synthesized consensus structures are shown in Fig. 1B, with their calculated size, solubility and electronic characteristics listed in Table 1.

NH-toc was generated by coupling a hydrophobic phytyl tail to a 2-aminomethyl-phenothiazine head group, to obtain a non-saponifiable compound with similar size and geometry and very similar hydrophobicity characteristics as tocopherol (Table 1). NH-trox was generated by hydrolysis of 2-cyano-phenothiazine, yielding a compound with essentially identical molecular weight and octanol-water partition as trolox. In contrast, NH-toc and NH-trox were characterized by significantly lower radicalization enthalpies (~27 kcal/mol) than their already remarkable parental structures (~31.5 kcal/mol). Concomitantly, the electronic energy levels of the lowest unoccupied molecular orbitals (LUMOs) of both NH-radicals were markedly lower than in tocopherol and trolox (by ~1 eV corresponding to ~23 kcal/mol) (Table 1). Both of these energy differences would predict an increased propensity of the NH-compounds to act as chain-breaking antioxidants [20].

3.2. Free radical scavenging activity of NH-toc and NH-trox

The potential of the newly designed compounds to directly interact with preformed free radicals was evaluated by determining their trolox equivalent antioxidant capacities (TEACs). The results in Table 2 demonstrate that NH-trox was superior to trolox both in aqueous and ethanolic medium. Similarly, NH-toc was approximately twice as potent as α- or γ-tocopherol in both solvents. As expected, the lipophilic phytyl group-carrying compounds were relatively disfavoured in the more polar, aqueous medium. Notably, all tested compounds possess the same number of scavenging groups, which is the factor that usually predominates in the experimental and theoretical explanation of differing TEAC values [9,27]. Hence, the higher TEAC index of the NH-compounds compared to their parental structures may indeed reflect a higher antioxidant activity due to electronic factors.

3.3. Inhibition of lipid peroxidation by NH-toc and NH-trox

The TEAC assay is in general of rather poor predictive value regarding the biologically relevant paradigms of oxidative stress protection, such as the inhibition of lipid peroxidation, towards which tocopherol has been optimized by evolution [1,29]. Hence, the effect of NH-toc and NH-trox was investigated in a native preparation of highly unsaturated rat brain membranes. It was found that NH-toc was significantly more effective in the inhibition of lipid peroxidation than α- or γ-tocopherol, affording comparable protection at 20- to 100-fold lower concentrations (Fig. 2). Commensurate with this finding, NH-trox was approximately 20- to 100-fold more potent than trolox. The efficacy advantage of the NH-compounds was noted with two unrelated analytical methods, i.e. the fluorimetric quantification of TBARS generation (Fig. 2A, B, E and F), and the immunological analysis of 8-isoprostane formation (Fig. 2C and D). Moreover, the advantage of the NH-compounds was found to be equally pronounced with two different prooxidant strategies, i.e. iron/ascorbate, which depends on endogenous, preformed peroxides (Fig. 2A–D), and iron/H_2O_2, which does not (Fig. 2E and F). These data indicate that the relative superiority of NH-toc and NH-trox is unrelated to the presence or absence of reduced ascorbate, the most probable regenerator of tocopherol in vivo.

3.4. Cytoprotective activity of NH-toc and NH-trox

To investigate whether the NH-compounds were able to translate some of their biochemical lead into the situation in living cells, cytoprotection assays in clonal hippocampal neurons were performed, using cell death as readout for cellular antioxidant failure. In this extended 24 h cell death assay (Fig. 3), all compounds possessing a lipophilic phytyl group, particularly α- and γ-tocopherol, were substantially more potent than in the cell-free lipid

Table 1
Calculated chemical properties of α-tocopherol, NH-toc, trolox, and NH-trox.

	Size		Solubility			Energy	
	M_w	α–ω [Å]	log*P*	*C*log*P*	p(head) [D]	ΔH_f [kcal/mol]	E(LUMOr) [eV]
α-Tocopherol	430.72	21.22	9.98	12.04	2.34	30.36	0.389
NH-toc	522.83	24.80	9.72	11.87	2.47	26.96	−0.633
Trolox	250.29	7.72	3.19	3.09	2.34	32.97	0.170
NH-trox	243.28	4.89	2.90	4.16	1.67	27.03	−0.964

M_W, molecular weight; α–ω, molecular distance between the reactive heteroatom and the solubility-determining side chain in a fully extended conformation (reading points are marked in Fig. 1); log*P*, octanol–water partition coefficient calculated by an autonomous fragmentation method; *C*log*P*, octanol–water partition coefficient calculated by a non-autonomous fragmentation method; p(head), dipolar moment of the antioxidant head group (boundaries are indicated in Fig. 1); ΔH_f, difference in the heat of formation of each compound and its radicalized state ("radicalization enthalpy"); E(LUMOr), electronic energy level of the radicalized state's LUMO (lowest unoccupied molecular orbital).

Table 2
Trolox-equivalent antioxidant capacities (TEAC) of the investigated compounds in two different solvents.

	Aqueous	Ethanolic
Trolox	1.00 ± 0	1.00 ± 0
NH-trox	1.73 ± 0.03	1.69 ± 0.11
α-Tocopherol	0.65 ± 0.04	0.80 ± 0.13
γ-Tocopherol	0.74 ± 0.07	0.87 ± 0.02
NH-toc	1.30 ± 0.10	2.74 ± 0.42

Data are expressed as mean ± SD ($n = 2$, each determined from four different concentrations).

peroxidation assays (Fig. 2). Nevertheless, NH-toc remained to be the most potent antioxidant with an EC_{50} value of ~35 nM, as compared to α-tocopherol with an EC_{50} value of ~150 nM. Trolox and NH-trox rather precisely retained their EC_{50} values from the iron/ascorbate-induced lipid peroxidation assay, without any loss or gain of activity in the cellular system. Hence, NH-trox remained to be ~50-fold more potent than trolox. The higher cytoprotective activity of the NH-compounds was not caused by higher cellular incorporation of these substances, as HPLC analyses indicated that NH-toc and α-tocopherol, as well as NH-trox and trolox were pairwise identical in their potential to permeate cells (Fig. 3D). Thus, it appears that the efficacy advantage of the NH-compounds in neuronal cells is indeed attributable to their improved structural ability to become stably radicalized and to so act as chain-breaking antioxidants. Notably, the advantage of extreme lipophilicity as conferred by a phytyl side chain seems to uncover only in cellular investigations.

4. Discussion

In the present study, we demonstrate that it is possible, based on the straightforward chemical calculation of only a few key

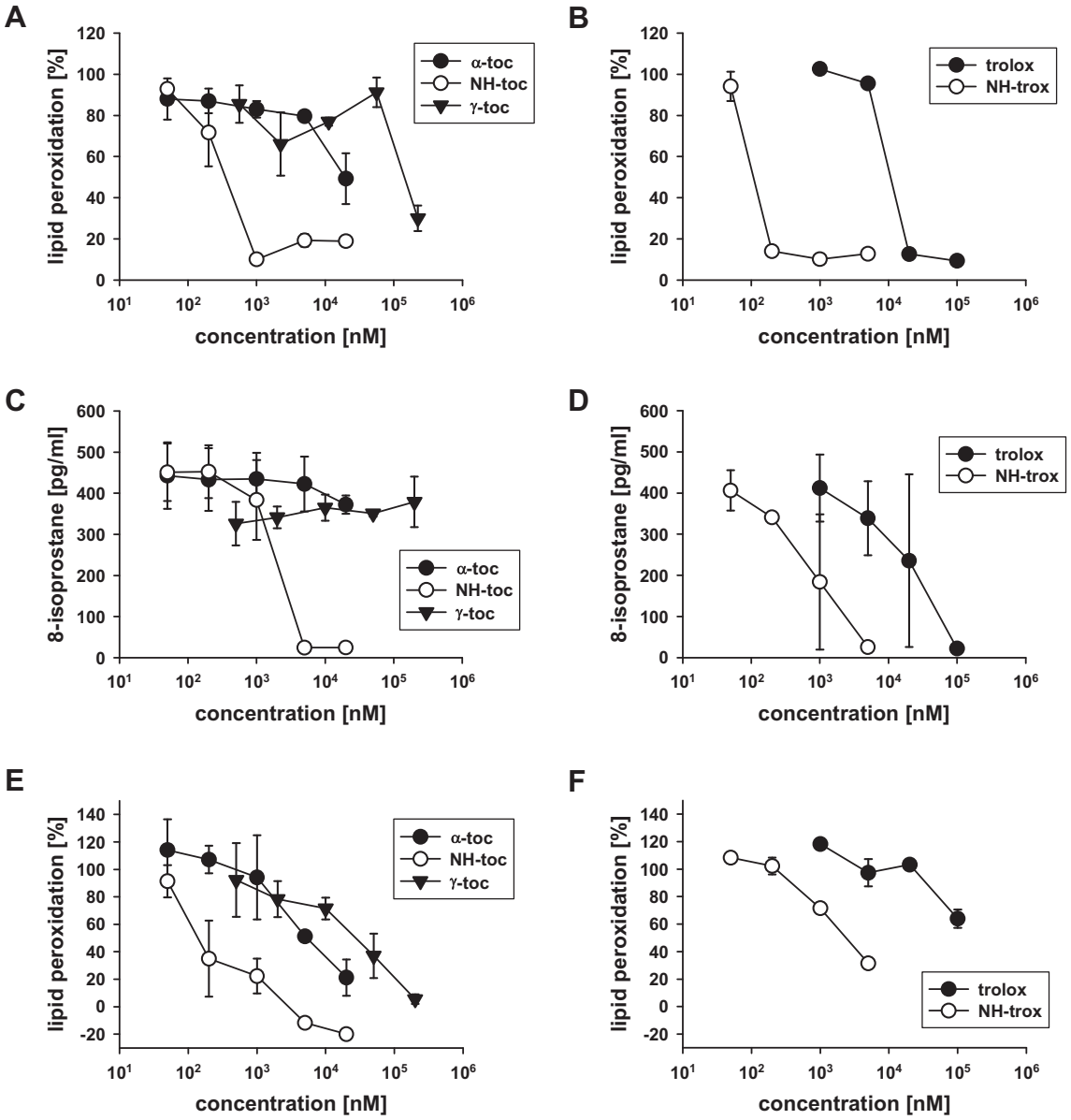

Fig. 2. Lipid peroxidation. (A) Activity of α-tocopherol, γ-tocopherol, and NH-toc to prevent general lipid peroxidation (TBARS generation) in a native preparation of rat brain membranes (0.5 mg/ml protein) challenged with 10 μM Fe^{2+}/200 μM ascorbate for 6 h. (B) Activity of trolox and NH-trox in the same assay. (C) Activity of α-tocopherol, γ-tocopherol, and NH-toc to prevent the formation of 8-isoprostane in rat brain membranes treated as in (A). (D) Activity of trolox and NH-trox under the same conditions. (E) Activity of α-tocopherol, γ-tocopherol, and NH-toc to prevent TBARS generation in rat brain membranes challenged with 5 μM Fe^{2+}/100 μM H_2O_2 for 10 min. (F) Activity of trolox and NH-trox in the same assay. All results are expressed as mean ± SD ($n = 2$).

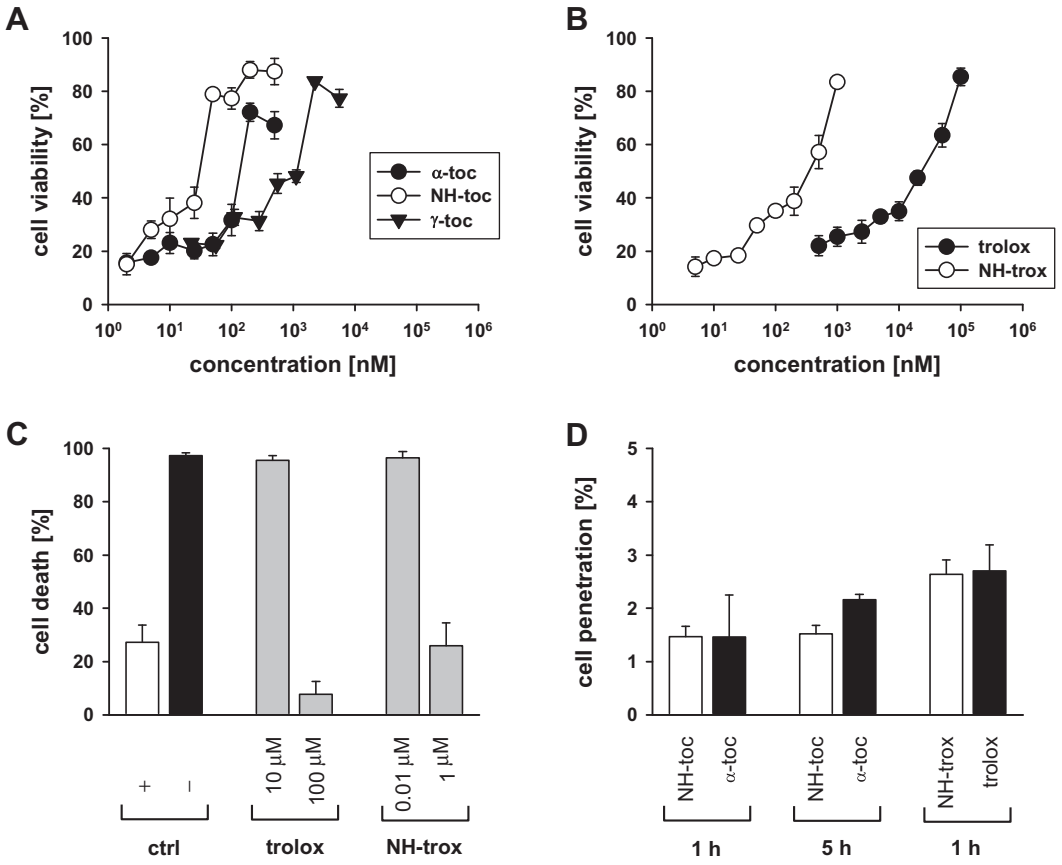

Fig. 3. Cytoprotection. (A) Effect of α-tocopherol, γ-tocopherol, and NH-toc on the viability (MTT reduction capacity) of HT22 cells challenged with 5 mM glutamate for 24 h. (B) Activity of trolox and NH-trox under the same conditions. (C) Cytoprotective activity of trolox and NH-trox in glutamate-challenged HT22 cells as determined by the PI exclusion test. (D) Cell penetration of α-tocopherol and NH-toc (10 µM), and of trolox and NH-trox (100 µM) after the indicated times of incubation. All results are expressed as mean ± SD (A–C: $n = 4$; D: $n = 2$).

parameters, to design chain-breaking antioxidants of superior efficacy than tocopherol and trolox. This conclusion is drawn from a number of assays that reflect the most relevant in vivo activity of tocopherol, the interference with free-radical chain reactions in non-polar environments. Given that tocopherol itself is a highly refined phenolic compound whose cytoprotective activity in cultivated neurons, for example, is unmatched by any other phenol, thiol, polyene, or nitrone [4,5], we speculate that aromatic amines may possess some inherent structural advantage with respect to potential antioxidant activities, and we conclude that this advantage is their better stabilization in the radicalized state when carrying two, potentially coupled, aromatic ring systems. This idea is supported by the fact that simple aromatic amines (such as 4-dodecylaniline) are just as potent as simple phenolic compounds (such as 4-dodecylphenol), while both of these structures are clearly less potent than diphenylamines [20]. Furthermore, the compound NH-toc was primarily designed as to obtain a close size and solubility match to its designated counterpart, tocopherol, and may thus not be optimized as an antioxidant yet. For instance, methylation or the addition of other substituents to the bare phenothiazine head group explored in this study may well result in an even better cytoprotective agent.

Beyond this merely biochemical consideration, it is clear that potential receptor-dependent mechanisms of authentic α-tocopherol or tocopherol's in vivo "vitamin E activity" in animals will not necessarily be mimicked by NH-toc, if just for the reason that without a suitable transfer protein as existing for α-tocopherol [29], NH-toc would probably exhibit a much less efficient resorption. In addition, chromanol and phenothiazine possess different degradation and excretion pathways, which might likewise influence their relative efficacy in experimental animals. Nevertheless, regarding their primary biochemical activity as lipid peroxidation inhibitors and cytoprotective agents, aromatic amines (here, phenothiazine) can in fact be much more potent than even refined phenols (here, chromanol). This raises the question why it was still the chromanol-headed compound tocopherol that was selected as nature's primary chain-breaking antioxidant rather than an aromatic amine, especially in view of the fact that biosynthetic pathways do exist for both classes of compounds [30,31].

Tocopherol synthesis in plants starts with tyrosine and proceeds via homogentisate, in which the later *para*-configuration of the two oxygen substituents of the aromatic ring is already preformed, to 2-methyl-6-phytyl-1,4-benzoquinone, a highly lipophilic isoprenophenol [31]. Tocopherol synthesis is completed by a ring closure reaction yielding δ-tocopherol, with possible methylation reactions resulting in β-, γ- or α-tocopherol. Intriguingly, each of these synthetic steps (lipophilization; ring closure; methylation) raises the antioxidant capacity of the resulting product, as detailed in the following. Tyrosine, a primordial mono-phenolic compound and proteinogenic amino acid, has been viewed as one of the oldest, if not the oldest chain-breaking antioxidant [11,32]. Tyrosine vastly gains antioxidant and cytoprotective activity when coupled with a lipophilic tail [11]. The ring closure reaction to yield a chromanol then enforces a molecular conformation in which the *para*-oxygen's lone pairs are specifically angled to further stabilize the radicalized state of the molecule [1]. Lastly, methylation especially of the phenolic *ortho*-positions lends additional inductive and steric stability to the radicalized phenoxyl state [12].

In view of this biosynthetic and evolutionary sequence of events, it appears that when nature was forced to develop better hydrogen-donating antioxidants due to rising ambient oxygen concentrations or other prooxidative changes in the environment, it was more opportune to stick to the once chosen structural motif and to gradually ameliorate the already available and approved phenolic strategy of antioxidation, rather than to shift concepts and to develop a totally new class of merely prospectively superior structures. This interpretation corresponds with the coevolution theory as pertaining to the origin of metabolic pathways [33].

The idea of coevolution is endorsed by the observation that the biosynthesis of aromatic amines generally depends on the anthranilate pathway, which is regarded to be a rather novel biosynthetic avenue as it predominantly provides tryptophan [30], the most recent of the standard proteinogenic amino acids [34]. Beyond tryptophan, only very few other metabolites descending from anthranilate have evolved, such as the acridone alkaloids [35]. Hence, it is quite possible that nature has just chosen phenols as prevailing chain-breaking antioxidants because the anthranilate pathway was not yet available when prooxidative changes in the environment demanded the development of a specialized, high-efficiency antioxidant, which tocopherol has become to be.

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